

Targeting androgen receptor activation function-1 with EPI to overcome resistance mechanisms in castration-resistant prostate cancer

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STATEMENT OF TRANSLATIONAL RELEVANCE

Androgen receptor (AR) N-terminal domain (NTD) contains activation function-1 (AF-1), which is a viable therapeutic target for castration-resistant prostate cancer (CRPC). AF-1 antagonists, such as EPI, have potential to overcome all currently proposed AR-related mechanisms of resistance. Compelling evidence indicate that gain-of-function mutations in AR ligand-binding domain (LBD) and constitutively active AR splice variant V7 (AR-V7) with truncated LBD are associated with resistance to abiraterone and enzalutamide. Here we demonstrate that EPI overcomes these clinically relevant AR-mechanisms of resistance that may drive CRPC that include overexpressed AR coactivators, gain-of-function AR mutations, and expression of constitutively active AR-V7. These results support the development of antagonists to AR AF-1 for the clinical management of CRPC. EPI is the first inhibitor that binds to the NTD of any steroid hormone receptor to be tested in clinical trials and is initially aimed at CRPC patients that have failed abiraterone or enzalutamide (ClinicalTrials.gov Identifier: NCT02606123).

ABSTRACT

Purpose: Persistent androgen receptor (AR) transcriptional activity is clinically evident in castration-resistant prostate cancer (CRPC). Therefore, AR remains as a viable therapeutic target for CRPC. All current hormonal therapies target the C-terminus ligand-binding domain (LBD) of AR. By using EPI to target AR activation function-1 (AF-1), in the N-terminal domain (NTD) that is essential for AR transactivation, we evaluate the ability of EPI to overcome several clinically relevant AR-related mechanisms of resistance.

Experimental Design: To study the effect of EPI on AR transcriptional activity against overexpressed co-activators such as SRC1-3 and p300, luciferase reporter assays were performed using LNCaP cells. AR-negative COS-1 cells were employed for reporter assays to examine if the length of polyglutamine tract affects inhibition by EPI. The effect of EPI on constitutively active AR splice variants was studied in LNCaP95 cells, which express AR-V7 variant. To evaluate the effect of EPI on the proliferation of LNCaP95 cells, we performed *in vitro* BrdU incorporation assay and *in vivo* studies using xenografts in mice.

Results: EPI effectively overcame several molecular alterations underlying aberrant AR activity, including overexpressed coactivators, AR gain-of-function mutations, and constitutively active AR-V7. EPI inhibited AR transcriptional activity regardless of the length of polyglutamine tract. Importantly, EPI significantly inhibited the *in vitro* and *in vivo* proliferation of LNCaP95 prostate cancer cells, which are androgen-independent and enzalutamide-resistant.

Conclusion: These findings support EPI as a promising therapeutic agent to treat CRPC, particularly against tumors driven by constitutively active AR splice variants that are resistant to LBD-targeting drugs.

INTRODUCTION

Most prostate cancers require androgen receptor (AR) transcriptional activity for survival and growth, and therefore, inhibiting androgen synthesis and AR transcriptional activity are the therapeutic approaches to treat advanced prostate cancer. These hormone therapies consist of androgen deprivation therapy (ADT) and application of antiandrogens. Unfortunately, within 12-33 months most patients treated with hormone therapies relapse with a more aggressive and lethal form of the disease referred to as metastatic castration-resistant prostate cancer (mCRPC) (1). mCRPC is associated with poor prognosis and a mean survival time of 18-36 months (2).

Despite ADT achieving castrate levels of serum testosterone, CRPC is associated with aberrantly restored AR transcriptional activity. The clinical onset of CRPC is characterized by a rise in serum levels of prostate-specific antigen (PSA), which is a gene regulated by AR. There are several possible mechanisms underlying continued transactivation of AR, including amplification of AR (3), gain-of-function mutations within the ligand binding domain (LBD) of AR that enables activation by nonandrogenic steroids or antiandrogens (4, 5), ligand-independent activation of the AR N-terminus domain (NTD) (6-9), overexpression of AR coactivators (10-14), intratumoral *de novo* synthesis of androgens (15), and expression of constitutively active AR splice variants with truncated LBD (16, 17). AR mechanisms of resistance to abiraterone and enzalutamide also include expression of constitutively active AR splice variants, elevated intratumoral androgen, and AR LBD gain-of-function point mutations (18, 19). Thus novel approaches beyond AR LBD inhibition are required to block AR transcriptional activity for the treatment of mCRPC. One such approach involves targeting AR NTD which would theoretically block all of the above AR mechanisms of resistance.

Activation function-1 (AF-1) in AR NTD is essential for AR transcriptional activity (20) and thus a viable therapeutic target for CRPC. An antagonist of AR AF-1, EPI-002 (2R, 20S) is a single stereoisomer of the EPI-001 mixture (21). EPI-001, its stereoisomers and analogues (referred to herein as EPI) all effectively inhibit the growth of CRPC xenografts in mice (21, 22). Specificity and the mechanism of action of EPI has been elucidated; it specifically binds to Tau5 of AR AF1 to block essential protein-protein interactions required for transcriptional activity of AR (21-24). Although at unusually high concentrations of up to 33 times its IC₅₀ and at a non-physiological pH of 9.4, EPI can have artifactual off-target effects (25). Here, we provide evidence that EPI can effectively inhibit aberrant AR transcriptional activity by mechanisms suggested to drive mCRPC such as overexpression of coactivators, AR gain-of-function mutations, and constitutively active AR splice variant. First-in-Human Phase 1/2 clinical trials with EPI-506 (prodrug of EPI-002) are ongoing in Canada and US (ClinicalTrials.gov Identifier: NCT02606123).

MATERIALS AND METHODS

Cell lines, transfection, and proliferation assay

LNCaP cells were from Dr. Leland Chung (Cedars-Sinai Medical Center, Los Angeles, CA). LNCaP95 cells were from Dr. Stephen R. Plymate (University of Washington, Seattle, WA). COS-1 cells were from Dr. Rob Kay (Terry Fox Laboratory, Vancouver, BC). LNCaP and COS-1 cells were not further authenticated in our laboratory, but were regularly tested to ensure mycoplasma-free (VenorTMGeM Mycoplasma Detection Kit, Sigma-Aldrich, St. Louis, MO). In September 2013, LNCaP95 cells were authenticated by short tandem repeat analysis and tested to ensure mycoplasma-free at DDC Medical (Fairfield, OH). All cells used were passaged less than 3 months after resuscitation. Transfection for luciferase reporter assays and cell proliferation assay were previously described (22). Details of transfection, proliferation assay, and plasmids and reagents are in the Supplementary Materials and Methods.

Western blot analysis

SRC-1, SRC-2 (TIF2), and SRC-3 (AIB-1) were probed with purified mouse anti-SRC-1, anti-TIF2, and anti-AIB-1, respectively (BD Biosciences, Franklin Lakes, NJ). p300 was probed using p300 antibody (C-20) (Santa Cruz Biotechnology, Dallas, TX). AR was probed by anti-androgen receptor antibody N-20 (Santa Cruz Biotechnology). Membranes were probed for β -actin using monoclonal mouse anti- β -actin antibody (ab8226 from Abcam, Cambridge, MA).

Protein-protein interaction studies

Cell-free SRC1-3 and AR interactions were analyzed on a Scintistrip microtitre 96-well plate (PerkinElmer Life Sciences) utilizing both purified recombinant AR-AF1 and *in vitro* transcribed/translated ³⁵S-labelled binding partners, SRC-1a-CTD (amino acids 977 to 1240), SRC2-CTD (amino acids 1120-1464) and SRC3-CTD (amino acids 1093-1412) (26). Cell-based immunoprecipitation assay was also employed to study SRC and AR interactions with details in Supplementary Materials and Methods.

Endogenous expression of genes regulated by AR

48 hours after treatment, total RNA was extracted by using RNeasy® Micro Kit (QIAGEN, Valencia, CA), and reverse transcribed to cDNA by SuperScript®III First-Strand Synthesis System for RT-PCR (Invitrogen™). Diluted cDNA and gene-specific primers were combined with Platinum® SYBR® Green qPCR SuperMix-UDG with ROX (Invitrogen™). Transcripts were measured by quantitative real-time (qRT)-PCR ABI PRISM 7900 Sequence Detection System (ABI PRISM®, Applied Biosystems by Life Technologies) in triplicates for each biological sample. Gene expression levels were normalized to housekeeping gene *RPL13A*. Primers have been described (22, 27).

Xenografts and animal study approval

Male NOD-SCID mice at 6-8 weeks old were subcutaneously injected with LNCaP95 cells (10 million cells per site) using Matrigel (Becton Dickinson, New Jersey). Mice were castrated when tumors volumes reached approximately 100 mm³. Drug treatment started one week after castration. Animals bearing LNCaP95 xenografts were randomized and administered with 100 mg/kg body weight of EPI-002 twice daily (b.i.d.), or 50 mg/kg body weight of enzalutamide

once daily (q.d.), or vehicle control (CMC/DMSO) q.d. by oral gavage. Tumors were excised 2 days after the last treatment. All animal studies conformed to the relevant regulatory and ethical standards. The University of British Columbia Animal Care Committee approved all experiments involving animals.

Steroid levels in xenografts and immunohistochemistry

Tumors were removed and 30-50 mg flash frozen for intratumoral steroid measurements as reported (18) and in the Supplementary Materials and Methods.

Statistics

Comparisons between two groups were performed with unpaired 2-tailed Student's *t* test, and comparisons between three or more groups were performed using One-way or Two-way ANOVA (GraphPad Software, V6). A *p* value less than 0.05 was considered statistically significant.

RESULTS

EPI maintains effective inhibition of AR transcriptional activity despite elevated levels of coactivators

Elevated levels of AR coactivators such as the p160 steroid receptor coactivator (SRC) family proteins (SRC-1, SRC-2, and SRC-3) and CBP/p300 are associated with prostate cancer progression and poor prognosis (10-14). SRC and CBP/p300 directly interact with AR NTD (8, 26, 28, 29). EPI blocks interaction of AR with CBP (22), but it is unknown if EPI also blocks interaction of AR with SRC. To test this, two approaches were employed. The first approach measured interaction in a cell-free assay with recombinant AR AF1 and SRC1,2,3-CTD proteins. This approach showed that EPI does not inhibit interaction between AF-1 and any of the SRC family members (Figure 1A). The second approach was to immunoprecipitate endogenous protein complexes from LNCaP cells with and without EPI treatment. These studies also showed no effect of EPI on this interaction (Figure 1B). Thus EPI does not block interaction of AR with the SRC family of coactivators. To test if EPI could maintain inhibition of AR transcriptional activity when SRC1-3 proteins were overexpressed, AR-driven PSA-luciferase reporter assays were performed in LNCaP cells transiently transfected with increasing amounts of SRC expression vectors. Consistent with previous studies (8), overexpression of each of the SRC family proteins resulted in a dose-dependent increase of androgen-induced AR transcriptional activity (Figure 1C, left panel). Western blot analysis confirmed the overexpression of each of the co-activators when compared to endogenous levels (Figure 1C, right panel). Despite the increase of androgen-induced AR transcriptional activity as a result of overexpressed SRC family proteins, enzalutamide and EPI both maintained effective and consistent inhibition of AR

activity, as indicated by the unchanged percentage of inhibition (Figure 1C, middle panel). Similarly, overexpression of p300 led to a dose-dependent increase of androgen-induced AR transcriptional activity in LNCaP cells (Figure 1D, left panel). Western blot analysis confirmed increased expression of p300 when compared to endogenous levels (Figure 1D right panel). Both EPI and enzalutamide effectively inhibited AR transcriptional activity in spite of elevated levels of p300 (Figure 1 D, middle panel).

Inhibition of AR transcriptional activity by EPI is not affected by polymorphic lengths of polyglutamine tract

The human AR gene contains a polymorphic CAG triplet repeat within exon 1 that encodes for a polyglutamine tract in its NTD . The number of CAG repeats ranges between 6–36 in the human population (30). Since EPI binds AF-1 in the NTD, we tested whether variable lengths of polyglutamine tract would affect the efficacy of EPI to inhibit AR transcriptional activity. AR-negative COS-1 cells were co-transfected with plasmids containing AR-driven probasin (PB)-LUC reporter and expression vectors encoding human full-length AR with polyglutamine tract containing 0, 12, 20, 40, or 49 contiguous glutamines. Regardless of the length of polyglutamine tract, EPI demonstrated consistent inhibition of androgen-induced AR transcriptional activity (Figure 2A, 2B). Consistent with previously published studies (30), these data confirmed the inverse correlation of the length of polyglutamine tract and AR transcriptional activity (Figure 2C). Western blot analysis revealed that levels of ectopic AR expressed in COS-1 cells were within physiological levels comparable with the endogenous AR level in LNCaP cells (Figure 2D). However, expression of AR with 49 repeats of glutamine (CAG49) was reduced compared to the other ARs with shorter tracts. Enzalutamide reduced the total protein levels of AR

consistent with previous reports (31). These results support that variable lengths of polyglutamine tract within the NTD did not diminish inhibition of AR transcriptional activity by EPI.

EPI inhibits transcriptional activities of AR with clinically relevant mutations

To assess the effectiveness of EPI against the transcriptional activities of several clinically relevant AR gain-of-function mutations including mutations found in both the NTD and LBD, we performed AR-driven reporter gene assays using AR-negative COS-1 cells transiently co-transfected with various AR mutants and treated the cells with different inhibitors. First, we examined two AR NTD gain-of-function mutations derived from patients treated with antiandrogens. AR E255K interacts with an E3 ubiquitin ligase and enhances protein stability and androgen-independent nuclear localization (32). AR W435L increases interaction between the AR NTD and LBD (32). EPI significantly inhibited androgen-dependent transcriptional activities of these two AR NTD mutations, and the level of inhibition achieved was similar to that of the wild-type (WT) AR (Figure 3A). This suggests that these NTD gain-of-function mutations did not impair the effectiveness of EPI to block transcriptional activity of AR. Next, we tested several AR LBD gain-of-function mutations. In the absence of androgen, EPI had no agonist activity which was contrary to hydroxyflutamide and bicalutamide that stimulated substantial AR transcriptional activities for WT AR and the mutants tested (Figure 3A). Enzalutamide also did not stimulate AR activity in the absence of androgen with any of these mutants. In the presence of androgen, EPI significantly blocked the transcriptional activities of WT, V715M, R761G, H874Y, and T877A (Figure 3A). Hydroxyflutamide failed to significantly block androgen-dependent transcriptional activities of AR H874Y and T877A, which is

consistent with previous reports that these two mutants confer resistance to flutamide (4, 33). No statistical significance was measured for the percentage of inhibition of androgen-dependent AR transcriptional activities between enzalutamide and EPI, with the exception of WT AR and AR T877A, in which enzalutamide displayed stronger inhibition (Figure 3B). Relative levels of ectopic expression of each of the AR mutants are shown in Figure 3C.

EPI blocks the non-canonical transcriptional program regulated by constitutively active AR splice variant

Expression of LBD-truncated and constitutively active AR splice variants AR-V7 or AR-V567es is associated with poor prognosis (17, 34). EPI inhibits the activities of AR-V567es in the presence and absence of full-length AR while antiandrogens, including enzalutamide, have no effect (21). AR-V7 is reported to regulate a distinct transcriptional program compared to full-length AR (35-37). Any AR LBD-targeting therapies including enzalutamide may not inhibit AR-V7's transcriptional activity and thus may have no effect on expression of AR-V7's transcriptome. However, an inhibitor of AR AF-1, such as EPI should block AR-V7 transcriptional activity and expression of its downstream target genes. To test this, the expression of genes regulated by full-length AR and AR-V7 was evaluated in LNCaP95 cells treated with antiandrogens or EPI. LNCaP95 cells express functional full-length AR and AR-V7 (16, 35). A PSA-luciferase assay confirmed that these cells do have a functional full-length AR that was responsive to androgen (Figure 4A). Androgen-induced PSA-luciferase reporter activities were inhibited by antiandrogens (bicalutamide and enzalutamide) and EPI. Consistent with LNCaP95 cells expressing functional full-length AR as shown with the PSA-reporter gene construct, endogenous expression levels of *PSA* and *FKBP5* were induced by androgen (Figure 4B). Both

enzalutamide and EPI decreased androgen-induced levels of PSA and FKBP5 transcript (Figure 4B). Next we measured levels of mRNA of genes that are distinctively regulated by AR-V7 such as *UBE2C*, *CDC20*, and *AKT1* (35). Firstly, expression of these genes was not induced by androgens thereby supporting that they were not regulated by full-length AR. Consistent with this interpretation, enzalutamide which blocks full-length AR, had no effect on the levels of expression of these genes (Figure 4C). Contrary to antiandrogens, EPI significantly reduced the levels of expression of *UBE2C*, *CDC20*, and *AKT1* (Figure 4C). Levels of mRNA for full-length AR and AR-V7 were not significantly affected by enzalutamide or EPI (Figure 4D). Nor were protein levels of full-length AR or AR-V7 decreased by any of the treatments compared to relevant controls and androgen treatments (Figure 4E). These data support the ability of EPI to inhibit the transcriptional activities of full-length AR and constitutively active AR-V7 to block expression of their target genes.

EPI attenuates the growth of enzalutamide-resistant LNCaP95 xenografts

Proliferation of LNCaP95 cells is androgen-independent and driven by AR-V7 transcriptional activity (16, 35). Therefore if EPI blocks the transcriptional activity of AR-V7 as suggested here then EPI should also impact the proliferation of these cells. Consistent with previous reports, proliferation of LNCaP95 cells was androgen-independent and resistant to antiandrogens such as bicalutamide and enzalutamide (Figure 5A). Consistent with EPI blocking AR-V7 transcriptional activity, EPI significantly reduced the number of proliferating LNCaP95 cells. Together these data suggest LNCaP95 cells are enzalutamide-resistant yet sensitive to EPI by a mechanism involving inhibition of the transcriptional activity of AR-V7. To test *in vivo* effects of EPI on potentially enzalutamide-resistant tumors, LNCaP95 cells were grown as xenografts in

male SCID mice that were castrated prior to treatments. EPI significantly attenuated the growth of LNCaP95 xenografts, whereas enzalutamide had no significant effect (Figure 5B). By the end of the study, animals treated with EPI had xenografts that were significantly smaller than those treated with vehicle or enzalutamide. EPI reduced the tumor size by more than 50% compared to vehicle control (Figure 5C, D), but did not result in tumor regression of LNCaP95 xenografts contrary to regression achieved with EPI in LNCaP xenografts (21, 22). These studies demonstrate the *in vivo* efficacy of EPI on the growth of castration-resistant and enzalutamide-resistant LNCaP95 tumors and support previous data showing the superior efficacy of EPI compared to antiandrogens on VCaP xenografts maintained in castrated hosts (21). Together these data are consistent with the notion that LNCaP95 tumors are driven by constitutively active AR-V7. Support for this can be provided by analysis of levels of expression of genes regulated by full-length AR and AR-V7 in the harvested xenografts. QPCR of RNA isolated from harvested tumors revealed that enzalutamide substantially increased levels of full-length AR and AR-V7 transcripts (Figure 5E), consistent with *in vitro* data (38), while EPI did not alter transcript levels of full-length AR or AR-V7. EPI treatment decreased levels of expression of genes regulated by full-length AR and AR-V7, including *PSA*, *FKBP5*, *UBE2C*, and *UGT2B17* (Figure 5E), consistent with the *in vitro* findings and gene expression data from VCaP xenografts maintained in castrated hosts treated with EPI (21). Measurement of androgen levels in these same xenografts confirms that LNCaP95 xenografts were not driven by androgen in castrated hosts (Figure 5F).

Immunohistochemistry analysis of harvested xenografts revealed similar levels of total AR protein with all treatments (Figure 6A). EPI significantly decreased proliferation as indicated by the lowered number of Ki67 stained cells, while it significantly induced apoptosis as indicated

by the increased number of TUNEL positive cells (Figure 6B). Consistent with TUNEL staining, cleaved caspase-3 staining of these xenografts also showed significantly increased level of cleaved caspase-3 in EPI treatment group (Figure S1). In contrast, enzalutamide had no effect on the proliferation and apoptosis in these xenografts. In accordance with previously published data that enzalutamide increases AR-V7 protein expression in LNCaP95 cells (35), we observed stronger nuclear AR-V7 staining with enzalutamide treatment, while full-length AR staining was similar between the treatment groups (Figure S2). Together these data support the efficacy of EPI in blocking the growth of castration-resistant and enzalutamide-resistant tumors by a mechanism that is consistent with blocking the transcriptional activities of both full-length AR and constitutively active AR splice variants.

DISCUSSION

Restoration of AR transcriptional activity is believed to be the major driver of most lethal mCRPC (39). Discovery of new drugs that block persistent AR transcriptional activities is crucial for improving the clinical management of this disease. In contrast to all current hormone therapies that act through AR C-terminal LBD, including abiraterone and enzalutamide, an antagonist of AR AF-1 in the NTD has the potential to block all known AR mechanisms of resistance. The present study revealed that EPI, an AF-1 antagonist, was capable of blocking such AR mechanisms of resistance. AR belongs to the steroid hormone receptor family that includes glucocorticoid receptor, progesterone receptor, estrogen receptor and mineralocorticoid receptor. These receptors share structural features such as LBD, DNA-binding domain, and an intrinsically disordered NTD. There are no small molecules reported that bind to NTDs of any other steroid hormone receptor. Thus EPI is precedent in the field of steroid hormone receptors as the first small molecule discovered that directly interacts with AR NTD (21, 22). AR transcriptional activity is dependent on a functional AF-1 region within its NTD which is comprised of Tau-1 (amino acid residues 110-370) and Tau-5 (amino acid residues 360-528). Tau-1 and Tau-5 may act independently of one another (20). EPI binds AF-1 (21, 22) and recent NMR spectroscopy has mapped its binding specifically to Tau-5 (24).

AR interacts with p160 SRC coactivators through both its C-terminal LBD and AF-1 (40-42) and with CBP/p300 in its NTD (29). CBP/p300 are bridging factors that are necessary for AR transcriptional activity (29, 43). The expression levels of SRCs and CBP/p300 are increased in CRPC and these increases in expression are proposed as an AR mechanism of resistance to current hormone therapies (10-14). Here, we demonstrated the ability of EPI to maintain an effective and consistent inhibition of AR transcriptional activity in spite of elevated levels of

these AR coactivators. EPI did not block interaction of AF-1 with p160 SRCs using recombinant peptides or with endogenous proteins coimmunoprecipitated from cells. This implies that p160 SRCs interaction with AF-1 cannot over-ride or compensate for the loss of interaction with CBP and RAP74 caused by EPI to decrease AR transcriptional activity (22). P300 and RAP74 interact with Tau-5 of AR AF-1 (24, 44). RAP74 is a subunit of transcriptional factor IIF (TFIIF) and an essential part of the basal transcriptional machinery to recruit RNA polymerase II that is necessary for transcription. Hence SRC interaction is not sufficient on its own to drive AR transcriptional activity in the absence of RAP74 and CBP/p300.

The length of polyglutamine tract influences interaction of AR with corepressors and AR transcriptional activity (45). The AR repressor, silencing mediator for retinoic acid and thyroid hormone receptor (SMRT), binds Tau-5 and LBD, and its ability to repress AR activity is highly influenced by the length of AR polyglutamine tract with only 17% inhibition of AR with 9 repeats and 56% of a AR with 42 repeats (45). Inhibition of AR transactivation by EPI was not affected by the length of polyglutamine tract within the NTD. EPI achieved significant and similar levels of inhibition of transactivation of AR with variable lengths of polyglutamine tract in the range of 0 to 49 repeats. The ability of EPI to continue to inhibit AR transcriptional activity in spite of differences in polyglutamine tract length is relevant to the clinical application of EPI to treat prostate cancer patients and particularly those patients with shorter polyglutamine tracts that correlate with more aggressive disease (46).

AR LBD is a structurally ordered domain with an open conformation for ligand binding, making the binding pocket for antiandrogen readily accessible (47). Contrary to this, the NTD is intrinsically disordered and acts as a hub for interaction with many proteins. Such differences in protein structure between the LBD and NTD may explain the rapid efficacy of antiandrogens in

short-term *in vitro* inhibition of AR transcriptional activity compared to EPI in reporter assays. It may take longer, perhaps one doubling time, in order for the binding site in the NTD to be accessible to EPI. However, in long term *in vivo* studies, the activities of antiandrogen and EPI were comparable in xenografts that express solely full-length AR in spite of the short half-life of EPI relative to antiandrogens (24). AR W435L (Tau-5) and AR E255K (Tau-1) are both cell-specific and reporter-specific gain-of-function AR NTD mutations that were originally detected using tissue from patients treated with antiandrogens (32). EPI effectively inhibited all the mutant gain-of-function ARs tested here, including E255K and W435L and several LBD mutations. The requirement of AF-1 for AR transcriptional activity supports a prediction that an antagonist to AF-1 should inhibit all LBD gain-of-function mutations.

EPI binds AF-1 (21, 22) and within this region specifically to Tau-5 (24), which is especially important for ligand-independent transcriptional activity of constitutively active truncated AR lacking LBD (20). Consistent with EPI binding to Tau-5, functional assays show that EPI blocks the activity of ectopic constitutively active AR splice variant and truncated AR1-653 deletion mutant (21, 22). LBD-truncated AR splice variants such as AR-V7 and AR-V567es are correlated with poor prognosis (34). Aberrant and persistent AR transcriptional activity caused by constitutively active AR splice variants is likely a main common mechanism of resistance to abiraterone and enzalutamide, and compelling evidence suggests that these variants provide resistance to current LBD-targeting therapies *in vitro* and *in vivo* (18, 38, 48). A recent clinical study revealed that the levels of AR-V7 in the circulating tumor cells from CRPC patients are correlated with resistance to abiraterone and enzalutamide (49). These findings indicate the potential of constitutively active AR variants to be a major mechanism of resistance to current AR LBD-targeting therapies, but more importantly, they highlight the need for

inhibitors of AR AF-1. In LNCaP95 cells, that endogenously express both functional full-length AR and AR-V7, EPI reduced the expression of genes regulated by full-length AR such as *PSA* and *FKBP5* and most importantly genes distinctly regulated by AR-V7, such as *UBE2C*, and *CDC20*, while enzalutamide had no effect. Androgen-independent proliferation of LNCaP95 cells was significantly reduced by EPI, while antiandrogens had no effect. Consistent with these *in vitro* results, *in vivo* EPI caused significant reduction of the growth of castration-resistant and enzalutamide-resistant LNCaP95 xenografts that recapitulate CRPC tumors driven by constitutively active AR splice variants lacking the LBD. Evidence for *in vivo*, on-target activity was provided by analysis of gene expression of the harvested xenografts showing EPI decreased expression of genes regulated by full-length AR and AR-V7. These results support previous findings that EPI specifically decreases the growth of castration-resistant VCaP xenografts that express AR-V7 splice variant with concomitant evidence of on-target activity provided by gene expression analysis (21). Thus, antagonists of the AF-1 such EPI represent a viable therapeutic approach to treatment CRPC tumours that are driven by AR splice variants, which have emerged as a clinically significant mechanism underlying the progression and therapy resistance in CRPC. EPI-506, a prodrug of EPI-002, is in clinical trials for patients that have failed abiraterone and enzalutamide with expression of AR-V7 proposed to be measured in circulating tumor cells (50).

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FIGURE LEGENDS

Figure 1. EPI overcomes aberrant AR transcriptional activity caused by overexpressed coactivators. (A) SRC1 and SRC3 show robust binding, while SRC2 shows relatively weak interactions with AR AF1. EPI-001 does not impair the binding of SRC1, 2 or 3. The inactive analogue compound 185-9-1 (B2H) was a control. The results are for two independent experiments measured in triplicate: SRC1 and SRC2 data was pooled (n=6), while SRC3 is a representative experiment (n=3). (B) EPI-001 does not block physical interaction between endogenous AR and SRC1 in LNCaP cells exposed to R1881 or vehicle (Ethanol). IP: immunoprecipitation; WB: western blotting. (C) LNCaP cells were co-transfected with PSA-luciferase reporter (PSA6.1-LUC) and coactivators. Cells were pretreated with vehicle, 10 μ M enzalutamide (ENZ), or 25 μ M EPI (EPI-002) for 1 hr before the treatment of 1nM R1881 for 48 hr. Luciferase activities were normalized to vehicle control treatment for SRC1, SRC2, SRC3, and (D) p300. Percentage of inhibition by treatments was plotted using R1881-treatment for normalization. Coactivator overexpression in LNCaP cells was confirmed by western blot analyses using specific antibodies with β -actin as a loading control. For the reporter assays, bar graphs are mean \pm SEM with n \geq 3 independent experiments.

Figure 2. EPI inhibits polymorphic AR NTD with variable lengths of polyglutamine tract. COS-1 cells were co-transfected with PB-luciferase reporter and expression vectors containing AR with different lengths of polyglutamine tract. Cells were pretreated with vehicle, 10 μ M enzalutamide (ENZ), or 25 μ M EPI (EPI-002) for 1 hr prior to treatment of 1 nM R1881 for 24 hr. (A) Luciferase activities for ARs were plotted in percentage of activation using vehicle-R1881 treatment as normalization. (B) Percentage of inhibition by treatments was calculated

based on vehicle-R1881 treatment and plotted. **(C)** R1881-induced PB-LUC activity under vehicle treatment was plotted for each AR. **(D)** The expression levels of ARs transfected in COS-1 cells were compared to LNCaP endogenous levels of AR by western blot using AR-N20. β -actin was a loading control. Bar graphs are mean \pm SEM with $n \geq 3$ independent experiments. One-way ANOVA compared the treatment groups to vehicle-R1881 control in (A), and ARs with variable length of polyglutamine tract in (C); * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 3. EPI blocks the transcriptional activity of ARs with clinically relevant gain-of-function mutations. COS-1 cells were co-transfected with PB-luciferase reporter and expression vectors containing wild-type (WT) AR and mutant AR. Cells were pretreated with vehicle, 10 μ M enzalutamide (ENZ), 10 μ M bicalutamide (BIC), or 10 μ M hydroxyflutamide (FLU), or 25 μ M EPI (EPI-002) for 1 hr prior to treatment of 1 nM R1881 or ethanol for 24 hr. **(A)** Luciferase activities were measured, and values were plotted after normalized to vehicle-ethanol treatment. **(B)** Percentage of inhibition by treatments was calculated based on vehicle-R1881 treatment and plotted. Bar graphs are mean \pm SEM with $n \geq 3$ independent experiments. One-way ANOVA compared treatment groups with vehicle controls in (A), and unpaired Student's *t* test was used to compare EPI with ENZA in (B); * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. **(C)** Protein levels of ectopic expression of WT AR and AR mutants in COS-1 cells compared to endogenous levels of AR in LNCaP cells were shown in a representative western blot using anti-AR N-20 antibody. β -actin was used as a loading control.

Figure 4. EPI inhibits transcriptional activity of full-length AR and AR-V7 in LNCaP95 cells. **(A)** LNCaP95 cells were transfected with AR-driven luciferase reporter PSA6.1-LUC and

pretreated with vehicle, 10 μ M enzalutamide (ENZ) or bicalutamide (BIC), or 25 μ M EPI (EPI-002) for 1 hr prior to addition of 1 nM R1881 for 48 hr. Luciferase activities were measured, and values were plotted after normalized to vehicle-ethanol treatment. **(B-E)** LNCaP95 cells were treated the same as above. Levels of mRNA were measured and quantified for **(B)** canonical AR-regulated genes *PSA* and *FKBP5*, **(C)** AR variant-regulated genes *UBE2C* and *CDC20*, and **(D)** full-length (FL) AR and AR-V7. Levels of expression for each gene were normalized to mRNA levels of *RPL13A*. **(E)** AR protein levels measured by western blot using AR-N20 antibody and β -actin as a loading control. Bar graphs are mean \pm SEM with $n \geq 3$ independent experiments. One-way ANOVA comparing treatment groups to vehicle controls; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 5. EPI inhibits the growth of CRPC driven by AR variants. **(A)** LNCaP95 cells were pretreated with vehicle, 10 μ M bicalutamide (BIC), 10 μ M enzalutamide (ENZ), or 25 μ M EPI (EPI-002) for 1 hr prior to addition of 0.1 nM R1881 for 2 days. Proliferation was measured by bromodeoxyuridine (BrdU) incorporation. Bar graphs are mean \pm SEM with $n \geq 3$ independent experiments. Two-way ANOVA comparing treatment groups to vehicle controls; **** $p < 0.0001$. **(B)** LNCaP95 tumor growth in castrated mice orally administered EPI (EPI-002) ($n=4$; 100mg/kg body weight, b.i.d.), enzalutamide ($n=3$; 50 mg/kg body weight, q.d.) or vehicle control ($n=3$; CMC/DMSO, q.d.) for a total of 26 doses. **(C)** Final tumor volume on day 27 prior to harvesting. **(D)** Photographs of representative xenografts harvested on day 27. The white scale bar represents 1 cm. **(E)** Transcript levels of full-length (FL)-AR, AR-V7, *PSA*, *UBE2C*, *FKBP5*, and *UGT2B17* normalized to *RPL13A* using total RNA isolated from the above LNCaP95 xenografts harvested on day 27. **(F)** Intra-tumoral steroid levels as determined by mass

spectroscopy, values are mean \pm SD in three tumors for each treatment. A significant decrease of testosterone and dihydrotestosterone following castration, $p < 0.001$, with no effects of EPI or enzalutamide on castrate steroid levels. There were no changes in dehydroepiandrosterone (DHEA), androsterone, or 5-androstenedione (androst-5-ene-3,17-dione, or AED) pre- or post-castration.

Figure 6. EPI reduces proliferation and increases apoptosis in LNCaP95 xenografts.

(A) Representative xenograft tumors stained for hematoxylin and eosin (HE), AR, Ki67 and TUNEL. Scale bars (red) indicate 20 μ m. **(B)** % of Ki67 and % of TUNEL positive cells were counted in xenograft sections of each treatment. Total number of cells counted: 2361 (Control, Ki67), 2658 (EPI-002, Ki67), 2409 (ENZA, Ki67), 1209 (Control, TUNEL), 1210 (EPI-002, TUNEL) and 1137 (ENZA, TUNEL). Bar graphs are mean \pm SEM with $n = 3$ different xenograft sections. One-way ANOVA comparing treatment groups to control; * $p < 0.05$; ** $p < 0.01$.

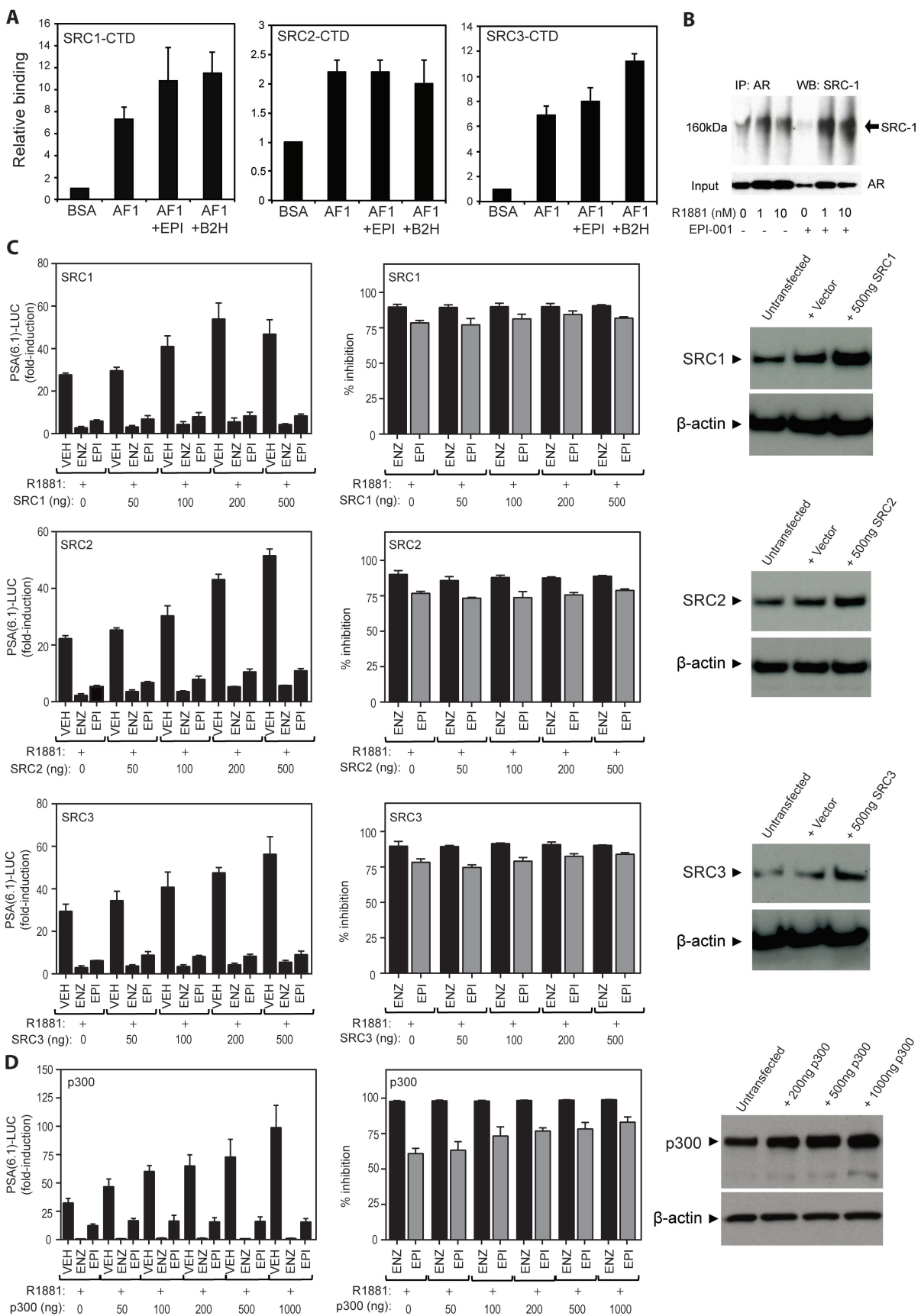


Figure 1

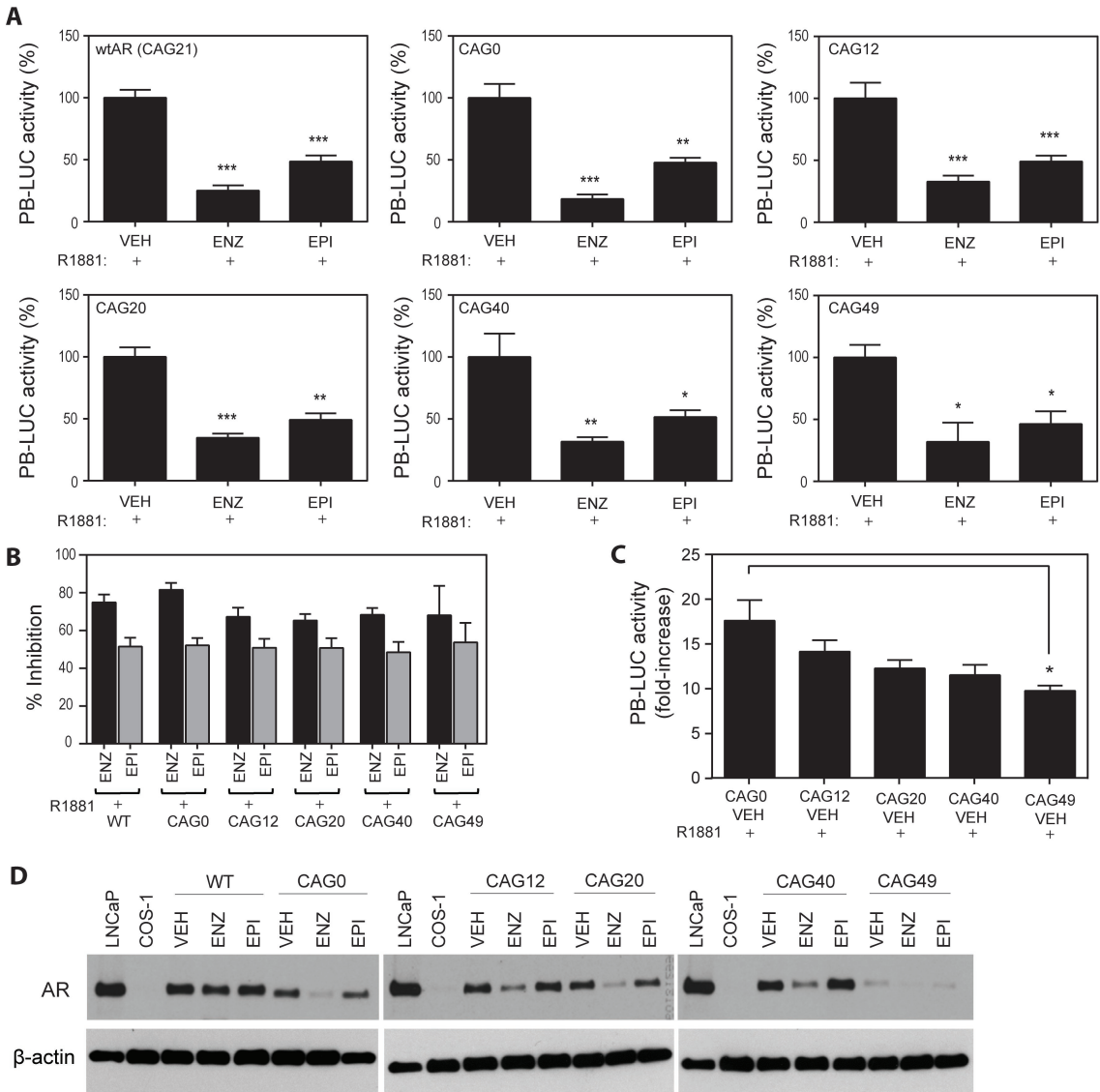
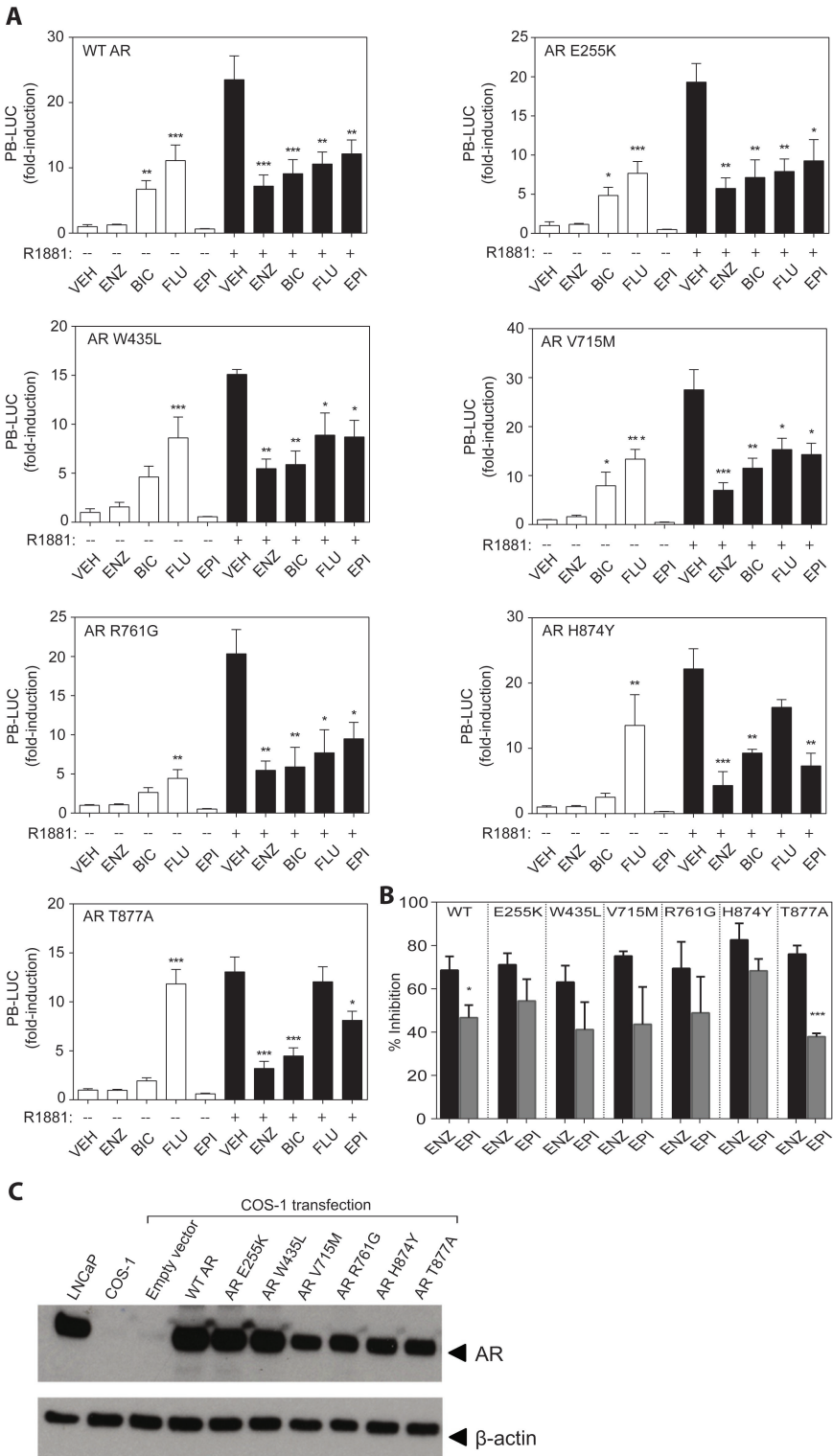


Figure 2



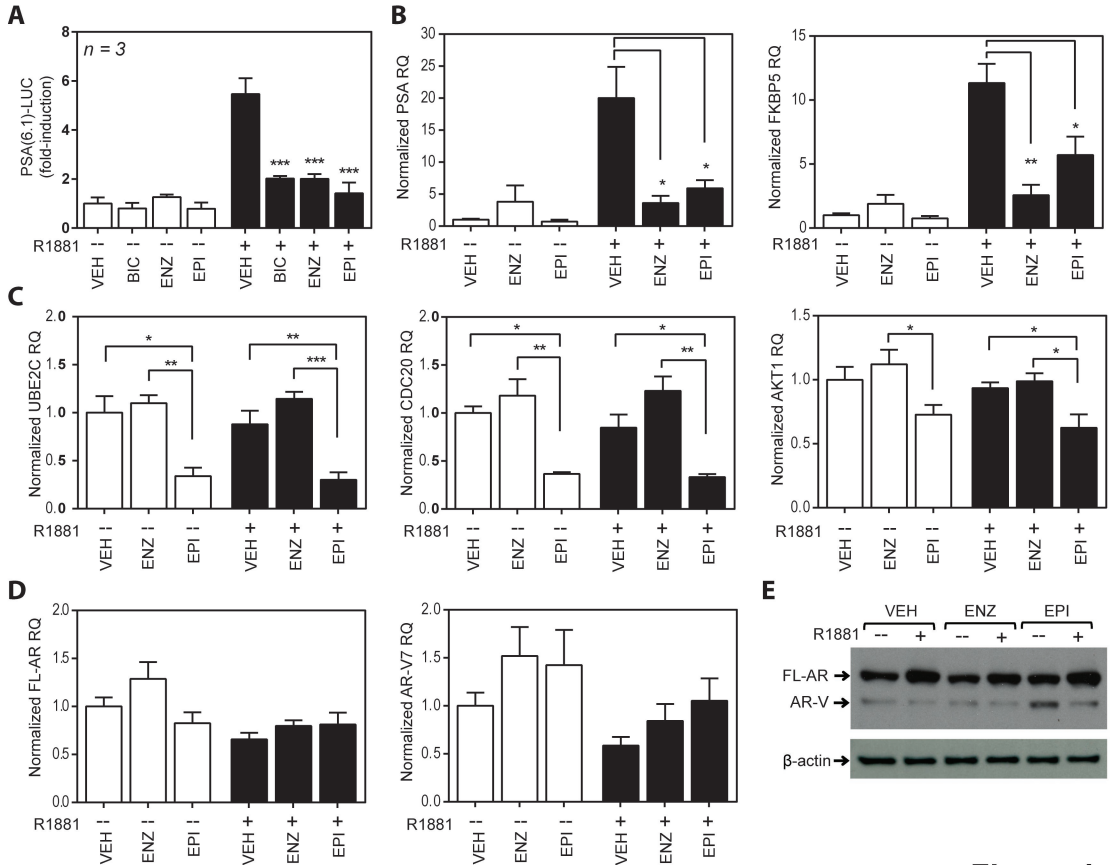


Figure 4

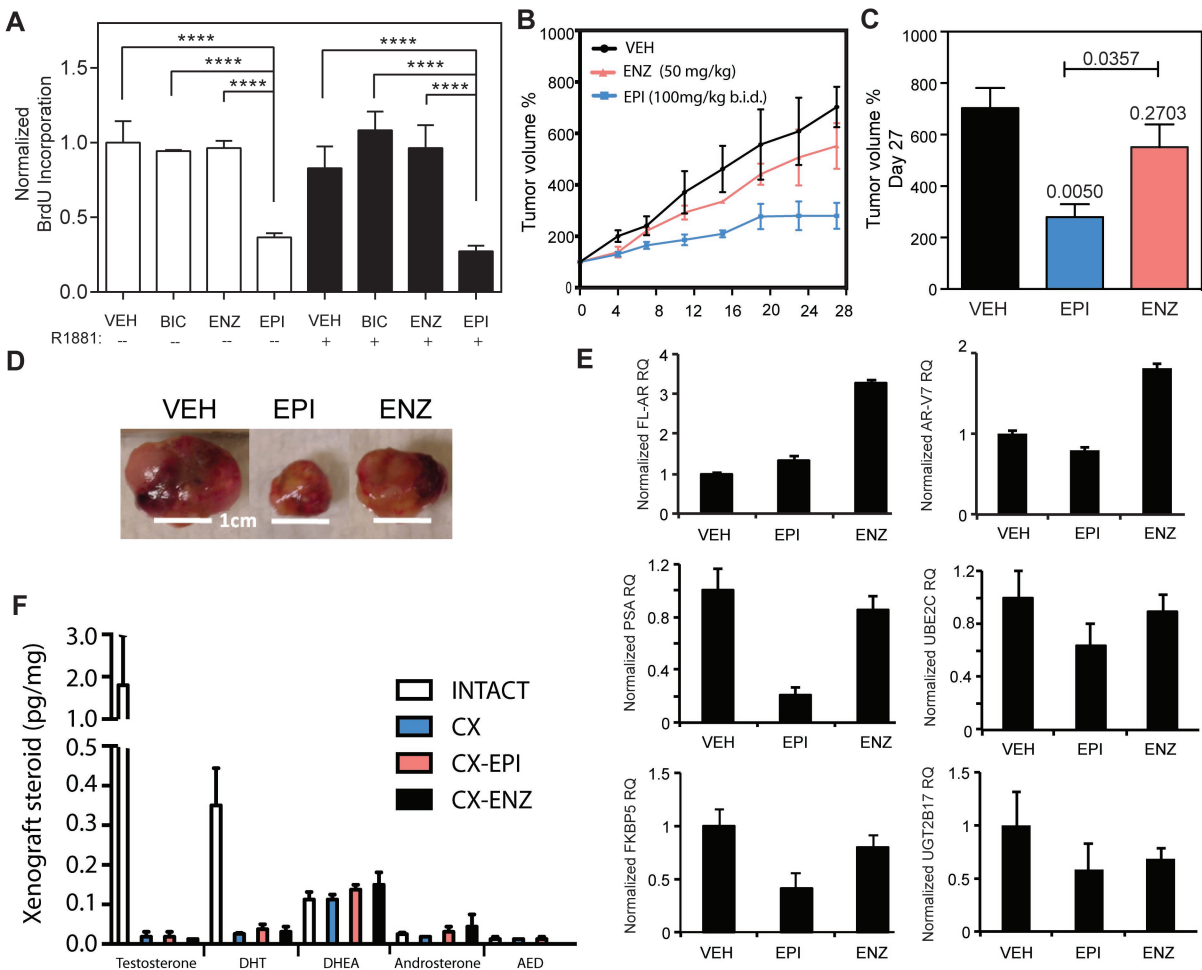


Figure 5

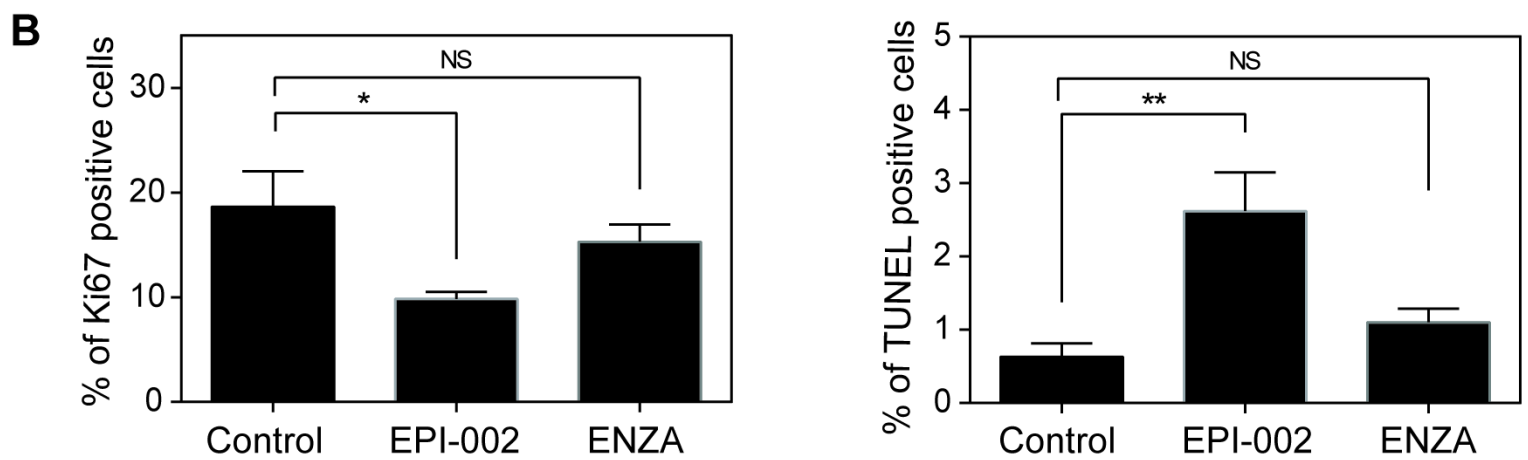
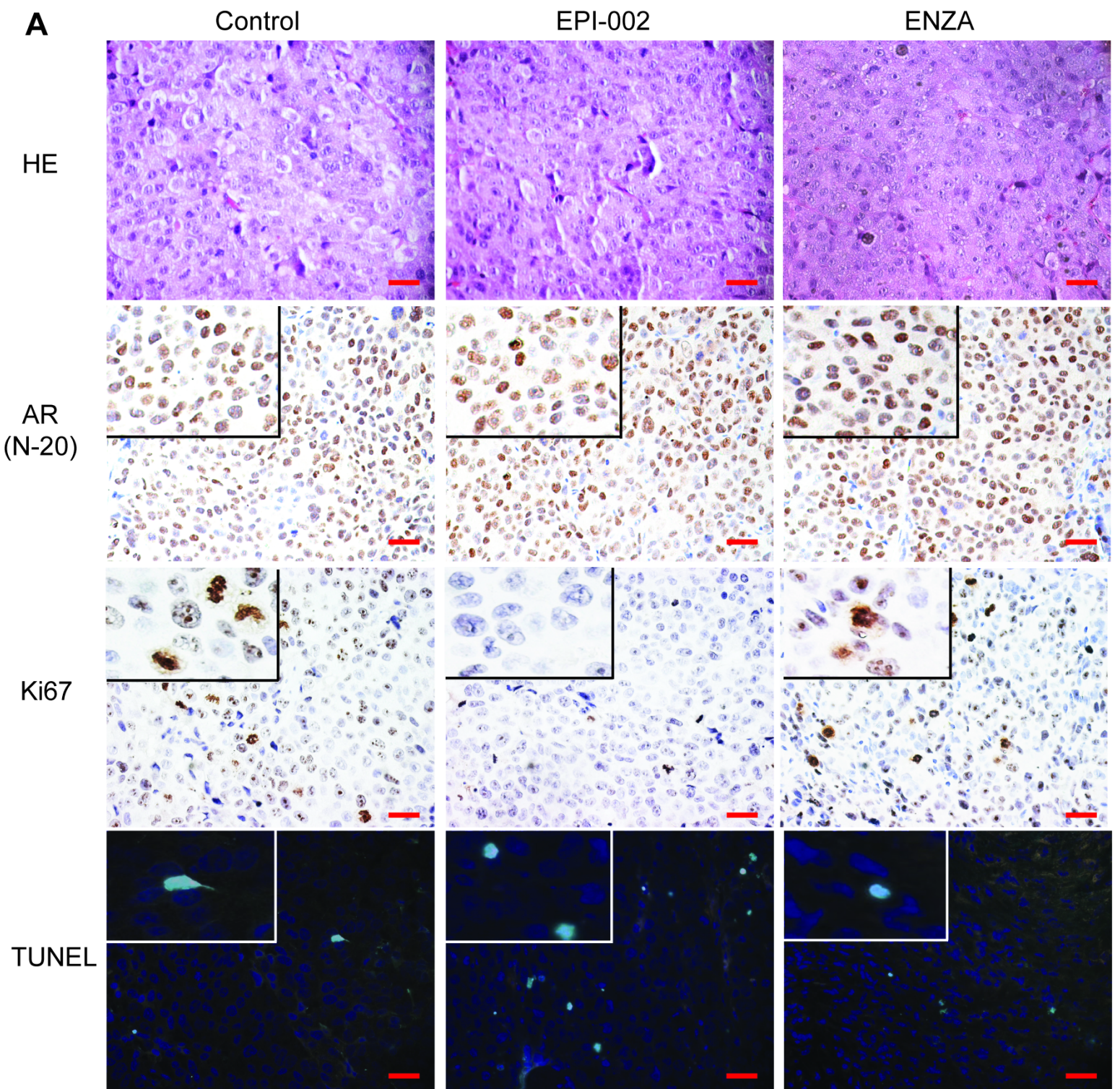


Figure 6